ANTIMICROBIAL RESISTANCE

PUBLIC MEETING

PRE-APPROVAL STUDIES AND PATHOGEN LOAD

STUDY CONCEPTS FOR MODELING RESISTANCE DEVELOPMENT

AND/OR PATHOGEN LOAD CHANGES

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I N D E X

STUDY CONCEPTS FOR MODELING RESISTANCE DEVELOPMENT AND/OR PATHOGEN LOAD CHANGES

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M O R N I N G   S E S S I O N

(8:40 a.m.)

CHAIRPERSON WHITE: I would like to welcome everyone back to day two of our pre-approval meetings. And we would like to welcome all of the new people that have joined us today as well. Today's meeting, the morning session is a continuation of yesterday afternoon's session and it is going to be focusing on study concepts for modeling resistance development and/or pathogen load.

We are going to have several speakers from pharmaceutical, academia, and government talk about their ideas about resistance development design and pathogen loads. We are going to have a break and then we are also to have a study concepts panel where the speakers from yesterday afternoon and this morning will come up on stage and maybe talk for a few minutes about the positive aspects of the model, what things they would change, what things we need to take into consideration.

Then after that we will have an open comment period where people can come up to the microphone. All I ask is when you go up to the microphone please identify yourself and the organization you are with.

So, are first speaker this morning is Dr. Susan Kotarski. She received her B.S. in Microbiology from Michigan

State University, a Masters in Microbiology from the University of Illinois, and a Ph.D. from the University of Illinois.

She then did a post-doc at Walter Reed in D.C. and she is currently a senior research scientist at Pharmacia & Upjohn. She is the project team leader and lead scientist to address antimicrobial resistance issues of a development of antibiotics for use in food-producing animals.

Please welcome Dr. Kotarski.

DR. KOTARSKI: Good morning everyone. I would like to thank the Center for inviting me to speak this morning and compliment them on offering a public session so we can discuss these concepts.

I am going to go right to my talk this morning.

(Slide)

As I understand it, my objective for today is to review in vitro systems applicable to pre-approval studies. My emphasis is going to be on bacterial system modeling. Specifically, continuous cultures and the use of batch cultures and their potential applications.

(Slide)

In order to do that, I thought what I would do is start right out by talking about continuous models. I think many of you have heard about them but haven't been involved in working with these systems.

(Slide)

This is a systematic diagram of the system that we are using. It is among the more simpler of systems that can be used. A continuous culture basically can be defined by the use of a continuous input of nutrient flow into a culture system. The objective being to maintain a bacterial culture continuously in a steady state or semi-steady state of a cultivated growth.

This is in contrast to a batch culture system in which the nutrient content is defined. The organisms grow within a finite volume. They have a build-up of toxicological end products. The organisms can die in this batch system as a result of the build-up and the depletion of nutrients. In the continuous culture on the other hand, there is a continuous influx of medium.

Now, in our system we are using a set of pumps for input of that medium and for removing the effluent to maintain a constant volume in that continuous state. We are also using an anaerobic system.

And this is the same system -- we are fortunate enough to have a number of microbial ecologists in our group that have devised a system in which we have a battery of eight replicates, each with its own nutrient input system. And the model system that we have been working with is developing continuous cultures of fecal flora to model the colonic flora in humans.

(Slide)

Okay. Well, one of the nice features of these systems is that they can maintain a fairly high level of bacteria with a fairly diverse population. The colonic system that we are mimicking contains on the order of 400 species, in feces typically. And a population of 10^{11} or 10^{12} cells per gram.

With these continuous culture systems, whether we are talking about the ones that we have been working with or ruminal ecosystems, the organism load that you can maintain is on the order of 10^{9} or 10^{10} cells per mil. So, we are talking about two orders magnitude lower than what is in an in vivo setting. For example, in a ruminal in vivo setting or in a colonic in vivo setting.

And what I also wanted to point out is that with these systems, that by virtue of the fact that we are using a sterile nutrient medium, we are essentially defining the inoculum at the onset. We are using one sample inoculum. We have a finite gene pool if you will.

We have, as the system evolves to equilibration state, we have a model of one ecosystem. And that one ecosystem may or may not represent the in vivo setting that it began to mimic at the tangent in which we sampled, or the dynamics of the system that occur in vivo.

One nice feature of that system though is that the, as I mentioned before, we had a growth substrate for semi-continuous or continuous maintenance of those cultures. They do maintain a dynamic metabolic state. Another good feature about this is that you can add antibiotics continuously or at pulse-dose or semi-continuously.

And this system lends itself well to developing concepts of bug drug interactions similar to the types of concepts that Dr. Papich was talking about yesterday. This system could be used for that, either for organisms grown in pure culture or more complex bacterial groups.

I also want to emphasize as a major point of this talk though, is that the variability of these systems have not been very well defined.

(Slide)

Well, let me talk about one developmental test system that we have used that has some parallels to pathogen loads and what we have found out in working to develop a test system.

The objective of a test system was in fact to identify drug concentrations that would not have an impact on the microbial ecosystem that it mimicked, namely the colonic flora of human fecal bacteria. That was our objective. To define a no-effect concentration for drug antibiotics on an otherwise stable ecosystem.

We decided to use a fecal inoculum as I mentioned before, and for our reference drug to develop the system we decided to use clindamycin. Pharmacia & Upjohn produces clindamycin, so that was about one of the major reasons for choosing that.

And secondly, another major reason is clindamycin is well characterized with respect to its effects in vivo on the colonic flora and its potential to disrupt the flora and the potential for overgrowth of certain opportunistic pathogens, mainly clostridium difficile.

Clostridium difficile is not part of the normal flora of humans. However, upon destruction by an antibiotic, if it is clindamycin or another therapeutic that might be used, it can if it is present and if it is ingested, it has a potential to overgrow. And if that strain overgrows and it is toxigenic it can manifest itself clinically as pseudomembranous colitis.

So, clostridium difficile because it was not an indigenous part of the flora was a natural choice and clindamycin was an obvious drug choice for reference for developing this in vitro system to test for a no-effect drug concentrations on equilibrated flora.

(Slide)

Now, what I have here is a diagram, conceptually of what we expected to see in the model. In any one experiment that we set up, what we initially do is equilibrate the culture to get a stabilized dynamic and as we work with this we use an inoculum of fecal flora such that it does not contain clostridium difficile. Or at least it is below the detection limits.

And what I am plotting here, conceptually, is the log base of the C. difficile population in that continuous culture. So, first we go through an equilibration phase to a point zero of the experiment. And on day zero of the experiment, once we achieve equilibration as measured by total population, we also can verify that the enteric populations are -- excuse me, the facultative populations are below the total anaerobic populations in those fecal systems.

Once we achieve that equilibration, then in our model system we add clindamycin at a level, at a particular test level, and we infuse that on a constant basis for seven days. Which is typical of a drug treatment therapy. And one other point that I might make is that the carryover time in the system was one day.

Another important point of this experiment was is that we added C. difficile on a daily basis starting on day zero. And what is different for this experimental system as compared to other experimental systems to detect C. difficile overgrowth either in vivo or in other in vitro continuous culture systems was that we didn't use a bolus dose of C. difficile.

Characteristically and the parallel that we spoke of yesterday in the 550-815 studies is that you give a large bolus dose of the organism, of the challenge organisms. This is in contrast to the normal setting either for C. difficile in the human situation or salmonella, as we mentioned yesterday, in the animal setting. Usually these organisms, unless they are in a disease state, don't see this level of organisms.

We reason that it would be better off in our test system to in fact challenge daily with a low level of organisms that would be more representative of the environmental setting. And this concept, if we are going to do salmonella challenge studies, might be incorporated into in vivo settings. Or may be one, you might want to think about it.

What is another driver considering this is a potential, is that we were looking at -- we were titrating the clindamycin concentration that we would add on a daily basis and we reasoned that clindamycin, at high enough concentrations, can inhibit the growth of C. difficile.

So therefore, there could be concentrations that we might add that would inhibit the growth of C. difficile. And the if so, and if we only bolus dose say for example on day zero, even though we might effect or disrupt the gut flora, in fact we might not detect C. difficile overgrowth because we are likewise inhibiting the organism.

So, it made sense then to challenge rather than on one large bolus dose on one day to instead challenge at a level that was low on a daily basis. And thereby, if we had a concentration that disrupted the gut but it was at a concentration that inhibited the C. difficile concentration, you would expect that once that clindamycin concentration declined then there would become a point when the destruction was such and the clindamycin was such that it permitted growth of the organism.

Likewise, if clindamycin at low concentrations that permitted growth of the organism and disrupted the culture, you would be able to see overgrowth at an earlier setting.

So basically we expected to see a type one response during clindamycin concentrations at the lower levels and perhaps a type two response or a delayed overgrowth with this type of set up.

And the other concept as well is, is that you might see no response if there was no disruption of the clindamycin you would expect to have seen no difference in C. difficile overgrowth.

So, what I want you to do for the next slide is just remember the colors of type one and type two response. In fact, what you will see in the next slides is that we were able to identify a concentration in this test system that allowed overgrowth during the clindamycin administration. That was at 2.6 microgram per mil.

We also found a type two response at the 260 microgram per mil concentration of clindamycin. And that 260 microgram per mil concentration is the concentrations that you might expect to see in feces.

(Slide)

So, what I am demonstrating here is three separate experiments. I want you to focus on the first experiment. Here again is the 2.6 microgram per mil response, that is that type one that I spoke of. We have a delayed reaction after the clindamycin infusion has stopped and we get an overgrowth at 260. And then there was an intermediate concentration that we tested as well.

The no-effect concentration that mimicked the zero dose was 0.26 micrograms per mil, shown in red. And that was great. That comprised one experiment. In total that experiment took about one month to run and three associates with a battery of six vestibules.

We tried the experiment again. This time we wanted to know if -- at that time we were using 1,000 cells per mil on a daily basis as our challenge dose for C. difficile. We wanted to titrate down and find out what was the minimum number of organisms that we could use to detect a similar response.

As you can see the type one and the type two response again showed up. This is one inoculum with the different treatment groups. And what we identified in that experiment was that a 10 cell per mil concentration in fact would not pick up the response but the 100 and the 1,000 cells per mill appeared to do so.

Before we wanted to go forward, we wanted to see really how reproducible this type of system was. You can see from one experiment to the next the dynamics of the response that we were seeing was fairly reproducible and it made us quite happy.

In the next experiment what you see is a representation of two sets of fecal inoculum. We had four replicate vestibules with four different treatment groups: 0.0, 0.26, 2.6, and 260. And the second set the fecal inoculum are designated A and B.

And I wanted to emphasize as well we were using four inocula for inoculum A and a different set of four inocula for the total inoculum for the replicate B. An important point is here, is that these were run simultaneously and in only one set of the four replicates were we able to detect an overgrowth response at the 260.

In neither system were we able to detect the type one response. And with the other response variables that we measure, we also saw a lesser response in terms of total VFA, impact on total bacteria.

(Slide)

So, my point is that basically, if we start looking at these models and start to reproduce them, we are likely to encounter some variation. That variation could in fact be two inoculum as suggested in our last experiment, but we haven't gone through the examination to identify whether or not the test system that we were using, some unidentified aspect of the test system was in fact accounting for that variation.

Ultimately though your test system is defined by that inoculum as I mentioned earlier because we are having a constant input of a sterile nutrient medium after the initial inoculum. And as well, the equilibrated state that you do achieve in these in vitro test systems is ultimately dependent on the culture conditions and the nutrient system provided to that system.

If we think about doing gene transfer studies in these test systems, these same concepts apply in terms of variability and the finite concept of the inoculum. The genetic determinants that you put in the system is dependent upon your inoculum. It is on that first day. It doesn't include the genetic variation or the genetic input that you might, that the animals might, receive in terms of the types of protocols we are thinking about for this workshop.

This system does not incorporate the day-to-day variation in terms of the organisms that are encountered by these animals. Ultimately, the inoculum, once inoculated into a culture system takes on an evolution of its own. The good thing is that it sets up a stable ecological system in which we can tests concepts of drug bug interaction.

But, it does not necessarily mimic the bacterial flora that the sample represented when it was inoculated and we have no information to the extent to which it can mimic, and it has a high likelihood that it does not mimic, the day-to-day changes and variations that an individual animal will encounter or the population as a whole.

(Slide)

This following slide is not a continuous culture, but I just want to bring to your attention the in vivo setting. This is a set of data that was generated about 10 years ago by Denny Corpet and it is simply a plot of total E. coli found in the fecal flora of humans on a day-to-day basis.

These people were given first a normal diet and then switched to a sterile diet. And the point of emphasis is, is that the darker -- I am having a hard time with this pointer -- the darker lines represent the tetracycline resistant coliforms that were present when the humans were eating a non-sterile diet.

And as you can see, even with the change of the sterile diet it detected resistant coliforms which represent in fact how a small portion initially became even less then.

So if we think, going back to an in vitro continuous culture system and modeling these systems it is woefully deficit in terms of modeling the day-to-day infusion of resistant organisms.

(Slide)

And also, with respect to these in vitro systems, they do have a useful potential which I will talk about in just a second. But, there are some other concepts that we want to think about if we are going to use it for any specific objectives.

And that is that the ecosystems that we are thinking about in terms of this workshop, there is not just one ecosystem available either with the animals or in the production setting.

(Slide)

A good example of this would be in the ruminant of course, and an obvious example actually when you think about the ruminant animal. The ruminant of course being the large organism it is and harboring a dynamic population with a dynamic input of organisms associated with this foodstuff presents a myriad of changing microbes with respect to influx of genetic determinants of population diversity.

Yes, it does have an overall stabilization, but there is variation associated post-randomly and over the age of the animal. Within this animal then there is not only a ruminant microbial ecosystem, but a colonic one as well.

(Slide)

And, if we want to think about monogastrics as maybe a more simplified system in terms of ecosystems, I would challenge you to think again. Within the colon, and I mean the different portions of the colon of course you can expect to see different ecological systems.

And the cecum that essentially represents a blind sac where the influx of nutrients and the dynamics of metabolism will be somewhat different. And there is well, lesser colonizations of the upper part of the G-I tract.

(Slide)

Another topic that came up yesterday, but is also apropos in terms of thinking about in vitro ecosystems is the inoculum that is used with respect to the health state of the animals.

(Slide)

If we are talking about animals that during treatment might be housed in a hospital pen or if we are talking about animals that might be exposed transiently to antibiotics during a healthy production state, these will also influence ultimately the ecosystem that we might introduce into that once in vitro system.

Another consideration in terms of ecosystems and the number of ecosystems is the age of the animal. The neonate will have a microbial flora. That microbial flora will change as it ages and that microbial flora will have different population levels as it ages.

So, thinking again for an in vitro system as a model to mimic in an animal, we are essentially taking one point in time for whatever ecosystem we choose to use.

(Slide)

So, overall I would like to personally recommend these in vitro systems have a predictive capacity at this point in time that is really unknown. One point that I didn't mention is that obviously these glass systems do not incorporate concepts of hosts metabolism and obviously the metabolism may effect the potency of the drug that is introduced.

These systems are closed systems, they model one bacterial ecosystem. I can't say that enough. The gene pool is defined and the test variability has not been determined.

Another concept is that if you are able to identify a no-effect concentration in these glass systems, the question then becomes how do you translate that from microgram per mil to microgram per kilogram body weight as dose function.

(Slide)

So, given that is the state in terms of modeling pathogen load studies or the rate and extent of a resistance emergence, I am not really keen on using an in vitro system to do so.

(Slide)

I would say that as we begin to look at in vivo systems, and I recommend that we might think about these systems, I anticipate that through the course of the discussions we will probably identify that there is no perfect in vivo system as well.

(Slide)

And so, from that standpoint, I mentioned earlier that the in vitro system has as a disadvantage that it doesn't take into account host metabolism as an example. That is a disadvantage in the context of a pathogen load study. It is a disadvantage, but it is an advantage.

And if your objective then is to better understand mechanistically the dynamics of the interactions drug bug interactions, a microbial ecosystem where you don't have variability of host metabolism entering into your studies might be to your advantage.

And from that standpoint, then I would urge you to think of the in vitro system more in terms of the context of your study objectives. Now at this workshop we are not at a point where we have identified specific objectives and I hope that at the close we will come closer to that goal.

But, given the facts of the case at this state, I would rather like to think about the in vitro systems in terms of a tool kit as we identify those objectives.

(Slide)

And from that standpoint, let me go into just briefly concepts of batch cultures. I emphasize again, batch cultures have a finite growth substrate. They typically use short incubation times and bacterial metabolism changes with times because the nutrient source is not replenished, it is limited, and there is no removal of bacterial end products typically.

Nonetheless, these batch cultures systems, whether they be pure culture or whether they be complex in vitro systems do provide rapid screening devices to get again information that is useful in our overall drug evaluation.

(Slide)

Okay. With respect to a batch culture, typically we will think of pure cultures. But, there is also concepts that we can use in terms of a complex inoculum. When I say complex inoculum, I am talking about an intestinal content: excreta, litter, or manure as the inoculum with no nutrient addition and a short incubation period.

I mentioned before a glass tube systems can only achieve levels typically of 10^9 to 10^{10} cells per gram in which we are going to test a drug concentration. If you take these samples and incubate them for a short periods, two to three hours, aerobically or anaerobically as appropriate, you can maintain that diversity for a short period of time and at levels that the drug might see in an in vivo setting.

From that standpoint, those types of systems might be useful for mimicking bacterial diversity insomuch as it is a short period of time, but it can be useful for screening for drug concentrations that might disrupt an ecosystem which might be detected by changes in fermentation acids or the production of hydrogen or a number of different response variables.

These also might be systems that can be used for detection of rapid drug inactivation. For example, if a drug is inactivated simply by the fact that it binds irreversibly to complex matrices within the inoculum, such that it decreases its potency, this is good information to know.

(Slide)

Let's give you as an example studies that were done some 10 years ago regarding ceftiofur degradation in feces. Our initial observation in residue decline studies was such that we could detect ceftiofur residues in the intestinal content of treated cattle. The concentrations were on the order of 10 to 11 parts per million in feces. But we were unable to detect any microbiological activity.

This in the face of the fact that we could also look at the tissue concentrations of ceftiofur and show that there was active metabolites present. The question then became well, if we can detect those residues in intestinal content, why are we not able to see microbiological activity? And in fact, we were not able to detect it. At least to the detection assay of our potency assays.

So, what we elected to do was to do some short-term incubations in which we took fecal material. We diluted it minimally and then asked the question if you add ceftiofur to these fecal incubations, the bottom line is that in a short period of incubation, on the order of four to six hours, for an addition of say for example 80 parts per million, the decline in microbiological activity, as is measured by microbiological cylinder plate assay or by HBLC.

We were able to show that in fact that decline was quite rapid within the space of literally hours. We have been able to demonstrate that in other species as well, including humans.

(Slide)

Another concept -- one more thing before I leave that. I realize that most antibiotics are not going to have this property. On the other hand, the property of inactivation does apply to other molecules, for example the aminoglycosides or the flouroquinolones.

Now, the extent to which that occurs can be refined or better understood in the context of using these as matrices. Likewise, this can be used as a rapid screening device if we think about new molecules that we might want to screen for this characteristic. It is going to be difficult to find, but nonetheless we might want to use it.

(Slide)

So, another concept to think about in terms of in vitro systems and rapid screening is just to better understand the frequency of mutation within the zoonotic population. This is a classic type of experiment that can be done very quickly.

And initially, once a drug is under evaluation -- I am not going to go into the details of that.

(Slide)

And classically, a number of MIC tests have applications that many of you are familiar with. Namely, these MIC tests defined the spectrum of drug activity both in zoonotics and veterinary pathogens. Usually, we don't emphasize the activity against commensals, but certainly we can incorporate that early on in getting a better understanding of the potential for causing changes in the bacterial flora.

We use these MIC tests to define potency. We use it for our interpretive criteria for efficacy. Certainly to support clinical efficacy studies for pharmacokinetics and pharmacodynamics as Dr. Papich mentioned yesterday. They help us to define cross-resistance to other drugs. They help us to characterize strains that are isolated from pathogen load and resistance emergency studies.

This is typical of the types of studies Dr. Mevius presented to us yesterday. Likewise, we use these MICs to understand the distribution of MICs for any particular species of organisms whether it be a zoonotic organism or a target pathogen. We would be best to understand what that distribution is pre- and post-approval.

(Slide)

The use of these in vitro systems in terms of complex ecosystem models we can then use to screen for drug concentration. They have that potential either to drug concentrations that disrupt ecosystems or drug concentrations select for antibiotic resistance. We can use them to screen or confirm drug inactivation by ecosystems.

We can also model short-term drug exposure scenarios. Say for example, in a continuous culture system, whether we are evaluating a one-time dose, a pulse dose, a continuous dose, or --- of antibiotics. That is not to say that they are necessarily predictive, but we can better understand the principles underlying the observations that we might have in a more diverse or complex matrix.

(Slide)

The predictive capacity, I will emphasize again, is really unknown though in terms of pathogen load studies and extent and rate of resistance emergence for many of these models. They do not incorporate host metabolism, they only model one bacterial ecosystem.

The result is defined by the culture condition. The gene pool is defined by the inoculum and test conditions. The test variability has not been determined. And the extrapolation is difficult.

(Slide)

So, overall my conclusions are that the in vitro test systems provide useful but limited information regardless of whether or not you can type supporting the processes of lead findings or drug evaluations or drug registrations.

There is no in vitro predictive test that I am aware of that is in place right now to provide pivotal registration data regarding the effect of drugs per se on resistance emergence and pathogen load.

I thank you for your endurance. And I will entertain any questions.

CHAIRPERSON WHITE: Okay. We have time for one or two questions. Please go to the microphone.

DR. GOOTZ: Tom Gootz from Pfizer. That was a good talk Susan. I was wondering, in this system though one advantage is the fact that if we are involved here, we are trying to assess the safety of animal health drugs with respect to selection or resistance of campylobacter, E. coli, gut organisms, it is true that the sponsor should know how much of their drug is in the fecal matter of those use animals.

And a lot of that drug obviously has to be bound to fecal material. So that only a fraction, let's say .1 percent is actually bio-available as it passes through the gut. Your system actually would be very good for trying to look at steady-state conditions at that level for each drug, for each agent, because that is probably the only system where you can look at population dynamics at concentrations of the drug that mimic that which is available in the feces.

So, that could be an advantage of this type of system. Have you tried -- as I noticed, just trying to look at some of your models, your data, when you have lower concentrations of a drug you tend to get less resistance. And maybe what we are often seeing here is this low-level free drug which is perhaps enough to induce certain types of resistance like to macrolide, but is not really efficient at selecting permanent mutations that cause resistance and then get into the environment.

Do you have any thoughts about that?

DR. KOTARSKI: A couple of thoughts. I agree with you that in this setting because you can define what your inoculum is you can look and try to develop model systems where you have low drug concentrations and look at the impact overall where you do have that complex ecosystem setting.

There are other models that have been used that are either plugged flow that involve matrices that are more complex than just a fluid matrix. We are using a fluid matrix here. Or they involve addition of substrates. For example, real food substrates.

You can have hardware that will accommodate that so you can incorporate not only the inactivation or binding that might occur for a drug to the bacteria themselves, but also other matrices that might be present that are not bacterial per se.

So, I absolutely agree with you. To develop mechanistic studies of bug drug interactions either in pure culture or in complex matrices, these have potential. Absolutely.

DR. SILLEY: Peter Silley, Don Whitley Scientific. Thanks for that Sue. Really just carrying on from that, have you looked in your model at actually putting any solid substrate into your system and seeing if there are any differences?

DR. KOTARSKI: We have not done that. I know that Karl Signiglia has a similar system and in fact we modeled our system on Karl's model in which he is using food substrates to dump -- excuse me, not dump in -- but add on a systematic basis, twice a day basis. I haven't seen the data that he's generated in that system with respect to this question, but I would anticipate that we will see some data coming from his laboratory in the future.

DR. SILLEY: We have been working with in effect a batch system which uses some solid matrices as well, and have been able to actually maintain from that a mixed fecal flora.

Certainly a reason to study --- in terms of the bacterial position that appears over 14/15 days. We have not really got any further than that.

But what is actually interesting, if you are looking at that sort of gene transfer and then you will actually see differences if you look in the liquid phase as opposed to actually in the solid phase, which is probably not surprising. But, I think it is quite interesting if we are then trying to maybe extrapolate to see what happens in vivo, because of course obviously there is a solid phase and a liquid phase. But there certainly are differences between the two.

DR. KOTARSKI: So, from what you are saying I understand that you can actually develop more than one ecosystem within that one culture?

DR. SILLEY: Yes. Absolutely.

DR. KOTARSKI: Okay.

CHAIRPERSON WHITE: One last question.

MR. : Susan, just a comment. You didn't highlight or perhaps we missed it, but the possibility of using the in vitro or lab-based studies to see the frequency of transferrable elements. You know, transconjucants, etc. like that which is something actually that might be quite useful and is done all of the time of course, if you know there is a transferrable element.

DR. KOTARSKI: Sure. Absolutely. I agree with you. I didn't want to highlight too much the pure culture work that can be done. In terms of characterizing the capacity for frequency of drug transfer, absolutely the pure culture work is useful. And it is also useful in terms of setting up systems to make comparisons to see that capacity pure culture versus seeing that capacity in a more diverse matrix.

Say for example like in an in vitro system such as ours. And that is easier to set up in an in vitro system than it is in vivo. On the other hand, the next question becomes, with that observation is how do you translate the in vitro observation to a capacity or put it specifically in terms of what is the rate of resistance emergence. That is difficult stuff. You know, translation of that in vitro data to in vivo data.

CHAIRPERSON WHITE: Thank you, Sue.

Just to let people know, we do have chairs in front. If you want to hold up the wall back there, that would be great too. The next speaker is Dr. Thomas Shryock. Tom got his B.S. in Biology from the University of Toledo and his Ph.D. in Medical Microbiology from the Ohio State University.

He then did a post-doc with Case Western Reserve University. He has been a research scientist, a senior research scientist with Pfizer. He has been an assistant professor in the Life Sciences Department at Indiana State University. He is currently a technical advisor and research scientist in the animal science discovery and development research program at Elanco Animal Health.

He also chairs -- I will keep going for Tom here -- the new Division C in American Study for Microbiology which is animal health microbiology. And he is currently the chair also the Veterinary Antimicrobial Susceptibility Testing Committee in NCCLS. Please welcome Tom.

DR. SHRYOCK: Thank you, David. Thanks to CVM for the invitation to participate in the pre-approval studies workshop. Unlike David's concluding slide yesterday with Apollo 13, I do agree that we do need teamwork, but susceptibility testing is not rocket science.

So, I hope to share some of the considerations that we will have to take into account, but keep in mind these are doable and certainly not that highbrow science that we would associate with Apollo 13.

(Slide)

With regard to the objective of the antimicrobial susceptibility testing or AST for shorthand, I think many of the previous speakers have already covered just about every point that I was going to make so I can roll through this fairly quickly. So, I will really try to emphasize some of the points that I think are essential to keep in mind as we enter into the break-out group discussions this afternoon.

Also, the handout that is available in the packet has many of these same points in it so you can refer to that as needed.

One of the key points to keep in mind is that while we do a lot of the susceptibility testing and relate that to in vivo outcomes, we really are making some arbitrary assumptions. In that the in vitro conditions are the in vivo conditions, and that is not true because we have host factors that also interact.

We can spend a fair amount of time on that triad then between host, bacterium, and antibiotic and we have to keep that in mind as we go through some of these discussions.

(Slide)

A number of the speakers that have already talked about the jigsaw puzzle as to how AST or susceptibility testing really is just the centerpiece to support a number of the other pre-approval studies.

For example, it has got a very significant role in clinical efficacy data that has a use for the PK/PD data as Mark Papich discussed yesterday. Post-approval we use this data for NCCLS guideline development. However, the data is actually developed during the pre-approval phases.

One thing that hasn't been mentioned is that all companies do some sort of field isolate survey for pre-existing resistance, usually in the target animal pathogen. However, I think with the recent discussions here some companies may be looking for some of the zoonotic to see what kind of resistance reservoir or potential exists out there.

Obviously, a spectrum of activity and potency are assessed on the typical battery of laboratory strains that companies have. All of this is used to phenotypically evaluate the resistance selection potential. And I will come back to that because phenotypic doesn't necessarily line up with the genotypic. That is still something that we need to put together in a much firmer way.

We can also use susceptibility testing to compare strains of known resistance characteristics. For example, there is quite a number of well-characterized resistance genes. A battery of those type strains can be tested to see what kind of response you will get with a new test agent.

And then lastly, the pathogen load and resistance selection studies that we are discussing today can also be very directly supported through susceptibility testing.

(Slide)

So, as I tried to put the talk together today I wanted to really try to list out some of the factors that I consider as I design susceptibility testing studies. As we do so we really come to the realization that a lot of this is an interactive consideration where we have to really know a lot about this type of specimen that we are going to be obtaining and how that interacts with how you isolate a pure culture, how do you identify it, to what level. Even some of the considerations on storage and perhaps shipping.

A number of speakers have talked about antibiotic properties, physicochemical characteristics that are important, as well as the actual methodology. All of that has to be factored in, in one way or another, in order to make an appropriate interpretation of the data.

(Slide)

With regard to the origin of the specimen, obviously this can take on many forms. Animal isolates from a variety of sources, either fecal, cecal tissue, etc. can be obtained at various time points throughout the life or medication period of an animal. I think that is very self-evident just by reading the slide.

Sue gave a very excellent talk on culture. Chemostat methods a sampling can be taken in those types of situations as well. And Kathy Ewert mentioned a battery of bugs yesterday, a reference culture type of situation. Those could also be considered an origin for your specimen.

(Slide)

As we continue on this path, recognizing that to do susceptibility testing we really need to, in my opinion, get down to some pure culture situations. Consider the type, whether it is environmental samples, fecal tissue, etc. and then that drives some of the sample size and processing-type of activities.

Dik Mevius mentioned yesterday that if you use 25 grams of fecal material versus one, your isolation rate goes up. Some of those kinds of considerations have to be given a little thought.

We talked a little about what kind of bacteria should we be looking at, we can discuss that in the break-out groups a little further. I think we have all got some fairly structured ideas on that.

Pure cultures, marked challenge strains for example, yeah those can all be done. Separating out mixed or contaminated cultures. There are some issues that go there. If you have an extraneous milieu, such as organic debris or blood that has to be taken into consideration.

And finally, a key point is the number of samples. Dik yesterday mentioned that if you have a culture plate that has 300 colonies, how do you choose which few or how many to take? Some real basic questions, but they do enter into the need for some consideration that does impact on the statistical design.

(Slide)

Obviously, isolation procedures are key in terms of trying to standardize and consider how you really approach your topic. Enrichment will pick up just one or two bugs, maybe as many as 10, depending on the specificity and the sensitivity of the enrichment.

That can only be varied also with selective enrichment where you may actually have antibiotics or other types of chemicals in there that really enhance the growth of a particular type of bacterium.

All of this leads up to the expected recovery rate. How many samples do you really need to take in order to get the desired number? If you need 100 samples and you anticipate you will get 40, that is a real factor there.

One thing that we have not really discussed is what about damaged cells or those that will be viable, but they are not cultivatable. That is kind of an unexplored area and something, again, just to consider.

(Slide)

Identification: how far down do you want to take that? Is the use of say, enterococci good enough? Or, do we need to speciate all of the various species of enterococci? Do we need to get into serotyping in some cases? Lots of considerations, lots of extra lab activity there.

(Slide)

As far as storage and shipping, some other considerations from real practical aspects. If you take the cultures you may want to test them all at once. It is a lot easier to do that. If you do that then there can be some other unforeseen affects. For example on gene or plasmid stability. Sometimes plasmids get lost, that can influence the interpretation of your data.

Obviously, recovery of pure cultures as opposed to mixed: sometimes you put away what you think is a pastorolida metastida and you get something else out, it happens. Not often, but it happens.

Viability: if you store these samples away, not all of them come back. And a lot of that has to do with the type of conditions that you choose to store your cultures in.

This also leads then to the next question of banking. How many isolates will you need to save for how long? How can you identify all of these things? It is not rocket science, but it is a factor that you have got to consider and order appropriate freezer or liquid nitrogen storage space.

There may be some limitations on biohazard agents as far as shipping or work in the lab that we might want to put forward.

(Slide)

Antibiotic properties is something that can be factored in fairly early in the game. Whether you are dealing with a novel class, one that is never been explored before or if it is an analog of an existing class, the extent of knowledge will vary.

Obviously, the physicochemical attributes, such as solubility, stability, potency, and purity requires a lot of chemistry support from the analytical and formulations group to help the microbiologists sort through that sort of thing.

Mark, yesterday discussed the mode of action: bacteriostatic and bactericidals, some other important characteristics to be aware of. We haven't really talked about testing related metabolites except in general terms, but that is something that could be considered. Especially if those have microbiological activity. We have already mentioned spectrum, narrow versus broad, and protein binding as a couple of other factors that could be considered.

(Slide)

With respect to the susceptibility test methods, there has been some question should we use NCCLS or should we use something else? That is a question that is certainly open and on the table.

The NCCLS manual here, which you can get from the NCCLS organization, basically is an SOP to do susceptibility testing and quality control development. There is another related document for sponsors that tells you how to set these types of things up.

This really is designed for the clinical, diagnostic laboratories. However, it can be used in research areas as well. So, while I am not going to spend a lot of time on this I just wanted to draw your attention that it can be useful, but there may be other methods that would suffice to address the research issues that are at discussion today.

No matter which of these methods, and they could include E-test, spiral plater, replica plating, filter methods, etc. always have to have some study to associate the conditions that affect the endpoint of those studies. Otherwise, you can get falsely high or low MICs.

For example, macrolydes are notorious for their Ph influence. So you have got to take that into consideration. For sulpha drugs, for example, the thymidine content in the media can influence the results tremendously.

Those sorts of things have to be considered and microbiologists are in a situation where they can do that, but all of this has to go toward the idea that you are going for validation, reproducibility, and the ability then to generate quality data.

As an aside, it was mentioned yesterday that we might want to consider testing human use antibiotics along with or in place of animal use antibiotics. I will just make the comment that the availability of the human use antibiotics may not always be there. Especially for companies for which there may be human-pharma competitive counterparts.

So, it may not always be practical to obtain some of those particular agents for comparative testing. At least in some cases.

(Slide)

Probably the key thing with all of the susceptibility testing data is what do you do with it once you have it? We can generate all sorts of numbers. Probably the most useful thing in the pre-approval mode would be the histogram frequency data. The slides that Dik Mevius shared with us yesterday would be a good example of that histogram frequency.

I think you can really start then to see some specific types of data and how that can be used to support some of your interpretations. That is as opposed to taking say an MIC 50, 90 or range, which is all right to do, but that is perhaps not the best research use or summary of the data in some of these types of studies.

For the interpretive criteria, which would be the susceptible intermediate resistant, that really has more to do with the clinical diagnostic laboratory utilization and interpretation of the data. And in part that is still related to pre-approval studies, but that is a little bit of a shift in emphasis.

Again, linkage that you have a phenotypically high MIC should be associated with the genotypic characterization for that resistance gene. The two do go hand-in-hand.

(Slide)

Just to list out some miscellaneous tests for susceptibility testing, these are things that you find in the literature quite frequently. For example, serum bactericidal activity attempts to look at the killing of the antibiotics in the presence of serum.

Obviously that doesn't hold for all drugs. For example those that are active against enterics. But, it does give you an idea of what happens in vivo in some cases.

Post-antibiotic effect can be measured quite effectively. It is a relatively simple and straightforward in vitro study to do. As well as subMIC effects.

All of these then tend to reinforce, because they are part of the PD characteristics, some of the uses of that whole discipline to support rational dosing designs. The same for kill curves and in some cases -- we really take this out a little bit further -- effects on virulence factors, for example toxin production, etc. could also be considered.

(Slide)

Some other considerations which may not be exclusively specific to susceptibility testing or laboratory activities include the logistics. Use of a single lab versus multiple can enter into this because of variability, various technicians doing different things at different times. So, that is factored in as I would design a study.

Obviously cost is a consideration. We have to be real about that. Validation, we have always got to justify that these methods that we are using are accurate, reproducible, and have some basis in fact.

Information technology or data capture and analysis, that is always a part of this but it is never voiced very openly. But that is a big part of it. You have got to take some kind of a record of your data and then do something with it. That is a consideration.

We haven't heard anything as far as statistical design on these experiments. Not being a statistician, I don't really want to get into that, but that is a very important consideration that we should pay attention to. Particularly, with sample numbers, statistical design, etc. because that forms the best basis for the interpretation of the studies.

If we are going to be using panels, obviously the manufacturer of those panels has some considerations there.

The last thing here to consider that hasn't been mentioned is all these studies need to be done under good laboratory practices which requires certain specifications that the lab must adhere to. It makes it a little bit more challenging, but by the same token it also makes the study that much more believable when you have these kinds of practices in place.

Ultimately, whatever protocol is decided has to be approvable by the CVM. That just goes without saying. That is all part of having NADAs and that sort of thing.

We haven't mentioned anything as far as in the pre-approval studies how that could relate to say a post-approval monitoring program. Certainly some of these studies that are done pre-approval would have to be looked at as foundations or background information to support some of the post-approval monitoring programs that will be considered.

(Slide)

So, to wrap up here and to complete the jigsaw puzzle. Again, to put into perspective, susceptibility data to me is the centerpiece. The interpretation is really key. At this point we are using the data for field surveys. Looking at reference collections. And sponsors are using the data also to develop the NCCLS guidelines and interpretive criteria.

They are also using these to support the efficacy studies and in some cases the rational dose selection through the use of PK and PD. The new piece of the puzzle would be the lower right-hand corner there (indicating) with the pre-approval studies and the resistance selection where we can actually then start to use these very same tools to apply in that regard.

(Slide)

So, once we have got the puzzle together we can get to work. And with that I will close and open it for any questions.

MR.   : Tom, could you explain what you mean that you sometimes have difficulty getting human drugs for susceptibility testing?

DR. SHRYOCK: Sure. The comment had been made yesterday that we might want to consider using human drugs to test for resistance selection. Obviously, if a veterinary pharmaceutical company is developing something that is in the pipeline in a different pharmaceutical company, the request may or may not be fulfilled by that competing company.

MR.   : You are talking about not approved human drugs, human drugs that are in development?

DR. SHRYOCK: It can be either one. I wouldn't necessarily limit it. There is no --

MR. : Well, I would take exception to that because one part of that is that there is no limitation. You can readily get, commercially get, human drugs that are approved to do susceptibility testing. I agree that if it is in human development it is hard to get. But, in actuality CDC as we -- as soon as a drug gets into Phase II trials, we often get the human drug to do susceptibility testing.

For instance, we are testing against zerosin which is in Phase III trials, etc. So, we can get the human drugs very easily, commercially even once it is approved. It is the veterinary drugs that we cannot get. The growth promoting veterinary drugs that we cannot get to do susceptibility testing.

So, I just find it ironic that you had a different conclusion.

DR. SHRYOCK: Tom?

CHAIRPERSON WHITE: One more question.

DR. GOOTZ: Tom Gootz. One of the hot button organisms is campylobacter. But I understand that there is not really an NCCLS approved or standardized, I guess I would say, method for susceptibility testing that. So, how dangerous is that for us to be doing a lot of pre-approval studies with campylobacter and even monitoring resistance if the NCCLS test isn't standardized yet?

DR. SHRYOCK: The NCCLS is working on that. Bob Walker could probably speak to this more effectively than I, but we are in the process of developing the quality control information for campylobacter against a variety of different antibiotics.

So, that should be available shortly. At this point, life does go on and people do use E-tests or other methods and come out with MICs. Chances are that those values will be fairly close to what the NCCLS comes out with. I can't foresee if they are going to be so different, that that would invalidate or repudiate existing data.

It would be nice to have some agreed-upon standards, and that is what Bob is working on and we should have those soon.

DR. WALKER: Yes. I think if you are going to be doing these studies, the things that we are looking at right now is the QC organism will probably be ATCC335660. So, campylobacter jejuni ATCC335660. Incubation conditions will be 5 percent CO_2 and the Mueller/Hinton broth for the microdilution, Mueller/Hinton auger with 5 percent blood for the auger dilution.

The disk diffusion, we are not going to recommend that testing method because it is like reading a hologram. It all depends on how you hold the platelet determines the zone size you get. Thirty-five to thirty-seven degrees centigrade for 48 hours.

DR. GOOTZ: If you --

CHAIRPERSON WHITE: Can you use the mic, sir.

DR. GOOTZ: Sorry. If you are to use an E-test on that in the interim for any macrolide and then incubate it in CO_2 to get the organism to grow, you might likely get much higher MICs since the acid nature of the CO_2 on the auger surface will artificially give you a higher MIC.

That is my only concern. But, no one is using that past this point. You said your standardized test is a --- broth?

DR. WALKER: Right. And we actually get better growth in 10 percent CO_2. The MICs don't change in 5 percent CO_2 or 10 percent CO_2, except for the macrolydes. And the macrolydes always have a higher MIC with the increased CO_2. So, what we are doing is developing the quality control ranges under these test conditions.

And so if you use this QC organism, use those test conditions, then any other test you run, as long as your QC organism is in control the rest of the testing method is, because you are defining those conditions, it should be okay.

CHAIRPERSON WHITE: Thank you, Tom. Our next speaker is Dr. Marc Lipsitch. Marc received his Ph.D. in Zoology from Oxford University. He did a post-doc with Dr. Bruce Levin at Emory University and he is currently an assistant professor of epidemiology at Harvard School of Public Health.

His research focuses on mathematical modeling and experimental approaches to study the population in evolutionary biology of bacterial pathogens.

DR. LIPSITCH: I am sorry, I am subject to Microsoft's file size inflation and wasn't able to get my entire presentation on a disk. It is not that long, it is just fat.

CHAIRPERSON WHITE: Just to remind people that we will have both a study concepts panel and an opportunity to ask questions at the end of this, after our break. So any of these questions can be revisited for our panel members.

DR. LIPSITCH: While we are waiting I will just tell you a little bit what I am going to be talking about.

As the introduction said, I am a population evolutionary biologist and have been interested recently in modeling antibiotic resistance in human community-acquired and hospital-acquired organisms. And, I don't have much first-hand knowledge about the farm animal situation, and am therefore going to try to talk about it to predict two models.

So we used, recently, to look at in one case a hospital-acquired pathogen and in another case a community-acquired viral pathogen. And try to show by example how those sorts of models can be used in a human situation and then try to draw parallels to where I think it might be useful in the animal area. And then describe some of the limitations as well.

So, we are getting close.

(Slide)

So, another outline of what I will talk about is I will briefly mention some mechanisms by which antibiotic use may select for resistance as those relate to the way those mechanisms are incorporated into mathematical models.

I will then describe how these models can be used to look at infectious disease transmission and give a couple of examples from our work. And then talk a little bit about the applications to the veterinary situation.

(Slide)

I have listed here three, what I think are distinct mechanisms by which antibiotic use may select for resistance. And which of these mechanisms is or are relevant depends on the organism and the drug that are in question.

And, I list them explicitly here basically because when constructing a mathematical models, one of the advantages is that you become explicit about your assumptions about the way in which your drug is related to your organism and to selection. And because of that advantage it is important to construct your model appropriately for the drug and organism in question.

So, in the human situation these are, the first of these is known as acquired resistance. The idea there is that a subpopulation is already resistant in the human host. Maybe a single mutant or a small population of mutants or perhaps organisms carrying a plasmid.

And that upon treatment the susceptible population declines and that resistant population overgrows, possibly leading to treatment failure. So, that is an event within a single host and it is a direct effect on the treated host.

A second mechanism is what is known as primary resistance. That is the idea that you can become infected directly with a resistant organism. That is why it is called primary resistance. And in this case, the use of the antibiotic, it is a sort of indirect effect in promoting resistance via primary resistance.

So, in this case the effective treatment of susceptible infections reduces the transmissibility of those drug-susceptible infections making it more likely that an individual who is infected will become infected by a resistant organism rather than a susceptible organism.

So the difference, there is an important difference there between primary and acquired resistance. Acquired resistance is associated with a failure of treatment, whereas primary resistance is really associated with a successful treatment. And in a way, the more successfully an antimicrobial reduces the pathogen population in a treated host, the more selection there is at the population level really on other hosts for the spread of resistance.

You will see what I mean by that later, I hope.

The third is via the effect on the normal flora so the treatment of one infection facilitates colonization by resistant organisms of another species via the effects on the bystander organisms. I think that is a process everyone here is familiar with.

(Slide)

The kind of mathematical modeling I am going to talk about today is what is known as compartmental modeling and I am going to show you in a little bit of detail what a compartmental model for a much simpler system would be like. Then I am going to tell you fewer details about my own models because they are a little more complicated than this and in the interest of time. But I want to try to make it clear what these models do and how they work.

They are called compartmental models because they track the progress of hosts, individuals, people in my case, through a series of compartments that refer to their state with respect to the infection you are interested in.

So for a simple infection, for example measles, you consider people born into a susceptible compartment. And then they may be infected at some rate moving them into an infectious compartment. And it is those infectious individuals who determine the rate at which new people become infected.

And then individuals recover at a particular rate and become immune, thereby making them no longer contribute to the process of making new infections. People are removed by these compartments either by aging past the age of susceptibility or, depending on the infection, or by death.

And you can model, for example vaccination, in something like this, in a model like this, by taking people directly from the susceptible category into the recovered or immune category without going through the infected state. And any model like this can be drawn as a diagram, but is represented in the analysis or the computer simulations that you do as a series of differential equations. And those are the equations for this fairly simple mode.

(Slide)

These kinds of models have been applied for a variety of questions. In the early part of this century models like this were used to evaluate interventions for malaria control. Comparing vector control for example, to preventing bites to various other interventions. They were targeted in different ways to ask which of those kinds of things would be the best to control the total burden of disease.

Another common use for these models is to calculate the critical coverage for an immunization program. So what fraction of a community has to be vaccinated by a particular vaccine in order to eliminate an infection from that community?

Another very interesting kind of application of these is to try predict and then prevent unintended effects of vaccination. The example for this is the story with congenital rubella syndrome. And I will talk about this just to illustrate how these indirect effects can work.

With rubella, the infection itself can be serious but the most serious public health concern, especially in developed countries, with rubella is the possibility of congenital rubella syndrome in which a pregnant woman becomes infected and consequences ensue for her child.

When you vaccinate against an infection, one of the effects is to make the average age in which that infection occurs increase. As a result of that, if you start with a community in which the average age of infection is long before puberty and you start to vaccinate, the number of people who are infected at puberty or post-puberty at a time when CRS may be a concern, will increase.

So, there is a possibility under certain circumstances for the actual burden of congenital rubella syndrome to increase as a result of vaccination transiently as the vaccine program is introduced.

That was recognized in the UK in the advance of the introduction of a rubella vaccination program. The program was then designed specifically in order to prevent that effect. So that is one of the really nice uses of this kind of modeling.

More recently, similar models have gained the spotlight in looking rather than at transmission in a community, looking at the dynamics of viruses in infected cells in individuals and showing the dynamic process of viral turnover in HIV infections.

(Slide)

So, how can these kinds of models be used to look at resistance? I think there are really two kinds of applications which may shade into each other, but really the approach is somewhat distinct and depends on the kinds of data you have and the kinds of questions you are interested in.

The first, which I will give an example of in a minute, is trying to make a very specific model to predict how fast resistance to one drug will emerge in a particular organism following a change in use.

And the reason why you can't always do this is because there is a requirement for fairly extensive data and a need to do fairly elaborate analyses of the uncertainty and the predictions associated with the uncertainty in your inputs. But sometimes that is possible and I will explain one example of that.

The other application is to use general models which are not going to give you an answer like resistance will double in 10 to 20 years or in 10 to 20 days. Instead, these models can be used to identify key processes and parameters in the transmission dynamics of a particular organism and the effect of the drug on resistance in that organism.

They can be used to suggest mechanisms that explain observations that have been made but for which the explanation has been uncertain. And they can be used to identify the approximate time scales for changes in resistance, even when precise predictions are not possible. I will give an example of that as well.

(Slide)

My first case study is a more predictive model. And this is a model that we put together in order to look at the changes in resistance to nucleoside analogs in herpes simplex virus type I, which is the cause of cold sores in a large number of people and a cause of a much more severe disease in immunocompromised people.

The maker of the topical cream for treatment, antiviral cream, for treatment of this infection, applied for over the counter status for this drug. This obviously raised serious issues of resistance. Particularly because the topical pencylovir cream showed cross-resistance to all of the major first line anti-herpes viral drugs.

So that if you select it for resistance to that cream it could quite serious, particularly in the smaller but important population of persons who got severe disease from the same organisms.

So we were asked to try to model the question what impact would increase use of topical pencyclovir have on resistance? So, there was a lot of data fortunately available for this situation. We knew a lot about the base line levels. We knew the current prevalence of the infection and of systematic recurrences of the infection.

We were able to calculate from these data that there is a period of roughly 10 years or more on average between transmissions. So that a person who is infected, on average it would be 10 years before they would pass on the infection, even if they were in a community of totally unexposed hosts.

We knew the current levels of anti-viral use and we knew that that was a large number -- and I am not giving numbers here because they are not so important in understanding the generalities here -- but, I can answer questions about that later.

They are large in kilogram and daily-dose terms, and it looked as though there was really a lot of use of the anti-viral class already. But, if you then looked at the proportion of the total cases being treated it was a very, very small number. There are a lot of people with recurrent herpes labialis and a very small number of those were being treated. So, as a selective pressure on the organism as a whole, the burden of current treatment was quite low.

(Slide)

And that was reflective in current resistance levels which were about .3 percent in the immunocompetent hosts and higher in the immunocompromised hosts, where the use was much higher and the emergence of resistance is much easier in immunocompromised hosts.

(Slide)

So, how does anti-viral use in this case select for resistance? Well, I described those three mechanisms and I think there are two of those mechanisms that are relevant for the HSV1 case.

The first is that treatment with the pencyclovir cream reduces viral shedding, the duration, by about 20 to 25 percent. And therefore may promote resistance by reducing transmission of the sensitive virus, leaving hosts susceptible to infection with resistant virus. Leaving the other hosts who might have been exposed to that susceptible virus, but aren't then susceptible to infection with resistant. So that is one mechanism and that selection is proportional to the efficacy of the drug in reducing shedding.

The other mechanism that might be involved is that in rare cases it may cause acquired resistance. And treated immunocompetent hosts were the ones to whom this OTC drug would have been marketed had it been approved, which it wasn't.

The data on that showed that in about 1,800 patients in different studies there were no clear reports of acquired resistance. There were four case reports, not in studies but just people who had been identified that might have been immunocompetent hosts in whom resistance emerged but it wasn't clear.

So, our best quantitative data was zero out of 1,800. Which if you take a confidence interval, the 95 percent confidence interval says that number can give you confidence that the true rate of acquired resistance is less than 1 in

625.

I am going through all of these numbers in this case for a reason. Because it turns out the 1 in 625 is actually very different from zero, which is what we initially thought. We in fact initially modeled it with zero and our predictions were so optimistic that we then wanted to see how close to zero is zero. And you will see that in a minute.

(Slide)

So we constructed a more complicated model than the one I just showed you which basically mimics that idea of people being born susceptible becoming infected. We put in two different kinds of infection: resistant and sensitive. We considered the dynamics of the way in which herpes works with period recurrences, some of which may be treated, thereby reducing shedding.

(Slide)

And we expanded that model. Again, our initial predictions were really very optimistic and we wanted to make sure that that was not an artifact of the model. So we expanded the model to include immunocompromised hosts and to include several other factors which we thought would be likely to speed the development of resistance in a population.

(Slide)

The uncertainties that went in the model were really parameter values. One was how much of this drug would be used? And based on parallels from the UK situation with another drug, we suspected up to 30 percent of episodes might be treated.

We didn't know, it is not known, how much of transmission comes from symptomatic versus asymptomatic sources. And that is important because if most transmission comes from symptomatic patients, then treatment of those symptomatic patients will have a big selective effect on resistance in the organism.

Whereas if most transmission is from asymptomatic patients then treatment of the symptoms may be much lesser of a selective force. We didn't know much about the immunocompromised in the transmission cycle. We didn't know whether resistant viruses are less transmissible than drug-sensitive viruses and we don't know the actual probability of acquired resistance.

(Slide)

So, as I said, we made some educated guesses and estimates of those parameters and we started by assuming no acquired resistance because we thought zero out of 1,800 sounds like zero. What we found that the scale, on the X axis there is years and the red line represents our most pessimistic assumptions about everything except acquired resistance.

So we assumed that all transmission came from symptomatic patients, meaning the selective effect would be greatest and we assumed that resistant organisms were just as transmissible as drug-sensitive. We made a variety of what we thought were pessimistic assumptions and the model predicts then a very, very small rise from about .3 percent to about .4 percent over 50 years.

So, that was wildly optimistic. We felt rather sheepish about this and a little uncomfortable and therefore we wanted to -- spent a lot of time trying to figure out what is accounting for this slow increase.

(Slide)

And we went back to this acquired resistance point. And I summarized most of what is on this slide earlier. The main thing being that we knew it was rare, but we didn't know how rare. And the numbers from studies that existed suggest that it was less than 1 in 600 patients.

(Slide)

So we then reran the model assuming that it was in fact 1 in 625 patients, which is at the top left (indicating). The slide I showed before is at the bottom right (indicating) for comparison and intermediate cases are shown in the other two.

And what you see at the top left is that making the assumption that instead of zero acquired resistance, it was 1 in 600 treated patients had acquired resistance, totally changed the dynamics of the system. And under the pessimistic assumptions, instead of going from .3 to .4 percent in 50 years, it went from .3 percent to almost 3 percent in 50 years.

And even under less pessimistic, more realistic assumptions in the yellow line, it went up to about 1.5 percent. Still relatively slow compared to some other pathogens that you may be aware of. But, the sort of wildly low numbers that we initially found were not verified once we added in acquired resistance.

(Slide)

So, what we concluded from that was that under all sets of assumptions the predicted increase in resistance would be slow compared to, for example, just to take an example of vancomycin resistant enterococcus where the doubling times have been in the year to two-year range and we have seen a really rapid increase over 10 years. We are looking at a few percentage points over 50 years.

But, we found that a small probability of acquired resistance dramatically accelerates the spread in the community. And we found that our conclusions were similar to predictions that were made a few years earlier by Blower and colleagues for genital herpes.

(Slide)

For a little point of comparison, this is the output of another model by Alan Pearlson's group in Los Alamos that looked at influenza A resistance under treatment in an outbreak with romantadine and amantadine. What they -- the dotted line at the bottom shows the resistance over this rather rapid breakout, over 30 days as the horizontal scale.

While the numbers aren't all that impressive in terms of total number of people, you see that the percentage resistance climbs rapidly and reaches a substantial fraction of the epidemic really within two weeks.

And so we wanted to understand of course why we are talking about a few percentage points in 50 years versus influenza with a couple of weeks.

(Slide)

And when we analyzed the differences between our assumptions and theirs, and then started tweaking those assumptions unrealistically in our case, but just to see if we could generate rapid increases in resistance like theirs, it turns out you can if change the parameters to be of a different disease.

And the parameters, such as influenza, the parameters that are most important in determining those, are first of all determining the rate of increase. First of all, the availability of resistant variance by acquired resistance or by gene transfer.

Secondly, the level of selective pressure. The acquired resistance as we saw made a big difference in our model and also the possibility for primary resistance in transmission between sensitives. Or reducing transmission of sensitives.

What is not important directly are things like kilogram usage, doses a little bit. But especially kilogram usage is not important. The compound we were studying, for example, was a topical compound and therefore the total amount of drug in there we calculated the price on a gram of drug basis and it is more expensive than gold as it turns out.

So that the total increase in kilogram usage compared to all the perentro use that was going on in the community would have been negligible had this drug been used at the levels contemplated under over-the-counter use.

Nonetheless, the selective effect would have been important because it was treating many more infections. So kilograms are not a very good proxy for selective effect.

(Slide)

The generation time of the infection is probably the most important difference between our results and the influenza results. In the case of influenza, the meantime between transmission is on the order of a week or two. In the case of herpes simplex infections type I, it is on the order of 10 years.

And that is basically -- natural selection works on the generation time of the organism involved. And in this case the relevant generation is not one viral replication, but it is the transmission of a case. And as a result, the time scale is magnified from weeks to decades in our case compared to the flu case.

Other factors include differences in transmissibility between resistant and sensitive infections and other factors which I am not going to talk about.

(Slide)

So, that is a case where we were able to make fairly quantitative predictions with quite wide uncertainty. But we were able to say even under the worst assumptions the rate of increase would not be rapid in the scale of weeks, it would take decades and it would be a few percentage points. Which might be very important and certainly clinically could have important consequences, but just as a matter of scale would not be the same as observed in some other infections.

What I want to turn to now is a second case study of some work we did on antibiotic resistance in hospitals. Which really tried to do a different thing. We weren't trying to make such specific predictions, but rather to explain some of the observations that had been made in hospital-acquired infections for which it was unclear the reason why.

One of those observations that had been made is that following an intervention in a hospital resistance levels can change much faster than they do in the community.

A second observation is that non-specific control measures, which are not targeted particularly at resistant infections can nonetheless reduce the frequency of resistant infections and maybe even more so than drug-sensitive infections.

And finally, there was a puzzling observation that in certain studies use of a single antibiotic could be a risk factor for carrying bacteria resistant to another. Even in the absence of cross-resistance or linked resistance determinants.

So for example, in a recent study flouroquinolone use was a risk factor for receipt of extended spectrum betalactam base gram-negatives in a hospital. And that is strange because what would the mechanism be?

(Slide)

So the first phenomenon I just described is a difference in rates of change between community-acquired and hospital-acquired infections. This is the data from a finish study supplied to me, kindly on a slide by colleagues at CDC, in which erythromycin use was curtailed by about six-fold in Finland.

And what happened following that was a gradual change in the levels of resistance to erythromycin in Group A Strep, first an increase, then a decline. Finally, about a 50 percent decline from the original level from about 14 percent to about 7 percent.

But that took from 1988 when the program was put in place to '96 when they stopped measuring. Over eight years we saw a relatively, well a 50 percent decline, over almost a decade. A similar situation was seen in strep pneumoniae in Iceland following interventions to reduce penicillin use.

(Slide)

And those are two of the real success stories that are cited for the benefits of controlling antibiotic resistance in the community, use in the community to control resistance. But in these cases the reductions take years.

Mathematical models by others, particularly Darren Austin and Roy Anderson at Oxford, have shown that the rate of decline in resistance is determined largely by the fitness cost of resistance. The difference between the transmissibility of drug sensitive and drug resistant infections.

Those models include some simplifications which may not be entirely appropriate. And the reason for my question mark under the comment "No fitness costs/no decline" is that I think there are ways in which declines can happen, even if there is no difference in transmission between resistant and sensitive. But, as a first approximation, that is expected to be slow or non-existent unless there is a fitness difference.

So, I think -- this is really directed toward another question, but the expectations for studies in the community, both previous experience and now mathematical models suggest that you should have had moderate expectations for the success of interventions to control resistance in the community.

(Slide)

In contrast to what you observe in the hospital, this is the result of a study from the early '80s on Methicillin resistant or Gentamicin resistant MRSA in a hospital. And as you see, following interventions in August of 1979, the level of this organism went from 30 percent down to zero in December and was really eliminated from the hospital.

(Slide)

So we wanted to understand what the mechanism of these differences between communities and hospitals might be. We constructed another compartmental model specific for a hospital. And, again I won't go into details, but the idea is that individuals could be either colonized with drug sensitive, colonized with drug resistant, or not colonized with a particular bacterial species which we did not specify because we were trying to do a general model in this case.

And, that individuals in contrast to a community where individuals are born not originally carrying a particular infection, or colonizing organism. In the hospital, individuals are admitted often carrying drug sensitive and sometimes even carrying drug resistant versions of the organism. E. coli or enterococcus for example. That turns out to be extremely important for the dynamics of the system.

(Slide)

So, the first prediction that we found was that using realistic parameters for a sort of compromise among several hospital acquired infections, you see very rapid response to interventions. If you reduce the use of a drug or switch it to another drug you see very rapid reductions in resistance to that drug over a time scale of days to weeks, in contrast to the community.

You also see that if you do infection control it disproportionately will reduce the prevalence of resistant bacteria as we observed.

(Slide)

And finally, we find a very strange phenomenon which is that, as I said earlier, sometimes in some studies if you look at the association between having received one antibiotic and having bacteria resistant to another you see a positive association. And that was a puzzling observation.

In this figure, we show -- the red lines show individuals who have received a second drug, drug two, and their level of resistance to a first drug, drug one, in those people. And the blue line shows those who have not received drug two.

And no matter what the overall rate of treatment with drug two, in that model we see that the persons who have received drug two are at higher risk of resistance to drug one. But, the more drug two is used in the hospital, at the whole hospital level, the association goes the other way. The more of drug two is used, the less resistance to drug one there is.

So, there are a lot of assumptions behind that which I am not going to have time to explain now. I am happy to discuss in more detail over coffee or during questions, but the point of this is simply to say that in a very simple system, it really matters what you measure. If you measure individual level associations or group level risks and that may have a lesson for other situations.

(Slide)

I went through the predictions rather quickly and didn't explain why. In all of those cases of those predictions, the reason for them and they reason why they are unique to hospitals, is this idea of people flowing through the hospital. Coming in colonized and staying for a short time and then leaving.

And this influx of drug sensitive bacteria makes a big difference to the dynamics of a hospital versus a community. And that may be relevant in considering the animal situation as well.

(Slide)

So, what can these kinds of models possibly suggest for animals, and I offer these tentatively in an audience full of people who know much more about the animal situation than I do.

First is population processes in the host population may be important. As I said, having an open system in which people are constantly coming in or animals are constantly coming in colonized may change the situation. And where the life span and the duration of stay in a particular group, like in a particular herd for example, has a big impact on the rates.

The second possible lesson is never say never. That comes from the herpes example. And what I mean by that is that very rare events like the one in 600 or less event of acquired resistance may have an important impact on the determinants of changes in the resistant level.

The third thing is that indirect effects are important. All of these models are really designed, and their strength is that they are good at looking at how treating some individuals affects the flora of others. And I think that if this kind of modeling can make a contribution to the farm animal situation, this is probably how.

And finally, models like this, even with limited data can give a rough idea of time scale thereby making a rational basis for measuring the effects of changes in antibiotic use.

While we don't know precisely what time scale we expect things to happen in a hospital, for example if you reduce the use of a particular drug, we know that the dynamics should be on the order of weeks to months, and not on the order of years. And that is done with very general data.

(Slide)

So, what can these models do? They can predict the response of bacteria to changes in antibiotic use if sufficient data are available. They can make testable predictions about the factors influencing these time scales, which analogy is the right one. So should we think of a particular agricultural or farm animal situation as being more like the herpes situation of decades or more like the influenza situation of weeks?

They can suggest mechanisms to explain previously unexplained observations and they can aid and study design by suggesting time frame, sample size, and some of the key processes that should be measured.

(Slide)

What can they not do? One thing, unfortunately, is they can't make reliable predictions based on limited data. The better your data, obviously, the more specific the predictions can be.

The second thing they can't do which I think is really critical in understanding the role of animal use of antibiotics and their possible impacts on human health is no model can predict very rare stochastic events which may be critical to the evolution of resistance, such as genetic innovations.

(Slide)

This is a dendrogram from a group led by Marshall and colleagues showing the Van-A, vancomycin glycopeptide resistance genes as found in enterococcus. Which groups, as being very similar in sequence to those from the glycopeptide producers, the organisms from which those drugs were initially isolated.

And how that jump was made, probably through other intermediate species is a mystery. And no model can predict that in any real sense because it is a rare event which just happened to take some decades probably, at least until it reached an important level.

And what role, use in animals plays in facilitating that sort of gene transfer is really, it is open to discussion, but it is not something that you can model.

(Slide)

And finally, I want to just make a brief comparison between what I have been talking about, my compartmental models, and the sorts of models that many of us saw presented. A very impressive example of last time we were here in December by David Foes, the risk assessment model.

The compartmental models that I have described are deterministic, while the other ones are probablistic or stochastic risk assessments.

One benefit of the kinds of models I talked about is that they are very good at looking at direct plus indirect effects, while the risk assessment models don't have population dynamics and therefore are much better for looking at direct effects.

Another benefit of compartmental models is that they naturally handle changes over time because of this dynamic aspect. Whereas the risk assessment models can do that, but it adds another layer of complexity.

On the other hand, the compartmental models have a harder time determining uncertainty because they are deterministic. You can add uncertainty analyses, but that increases the complexity. While uncertainty analyses is a quite natural part of risk assessment modeling.

(Slide)

And finally, I just wanted to mention my collaborators on the herpes work: Bruce Levin, Arista Mantia at Emory University and colleagues at Smith Kline Beecham. And in the hospital work: Karl Berkstrom and Bruce Levin at Emory.

Thanks!

CHAIRPERSON WHITE: We have time for one or two questions, real quick.

MR. : Marc, I think there are examples of some of the points that you've raised from agriculture and I think a really great example is the slime facility at the University of Kentucky which has been without tetracycline use for a decade, yet still has a prevalence of tetracycline resistance amongst E. coliform.

And probably because they are of course giving their replacement pigs from -- it is not an entirely closed -- and the second thing is, interest in data from both the United States and from Denmark and Europe of the transition of traditional dairies to organic dairies and how long it takes for resistance to be impacted as they change to an organic dairy, which organic dairies don't use any antibiotics.

And what is interesting is that until the organic farm -- experience in both those settings -- until the organic farm changes to raising all its own replacements, not having any -- purchasing any -- so in other words, to become a certified organic you have to raise your own replacements. But during that transitional period they are purchasing replacement heifers.

They see no impact upon resistance until they have a closed cycle.

DR. LIPSITCH: Right.

MR.   : In fact aren't bringing in external pressures that might apply. And just -- the last thing. We emphasize the idea of an unpredictable event is really the most disconcerting thing because I think as we -- those uncertain events, rare events are certain to occur, we just don't know how long it will take until they occur. So, although uncertain they are certain to eventually happen.

DR. LIPSITCH: I agree and thanks for both the suggestions of the data. I would be interested in learning more.

CHAIRPERSON WHITE: Any other questions? If not we will move to our break. We are about 10 minutes behind schedule, but we started 10 minutes late so being optimistic we are right on time.

So, why don't we -- we have a 25 minute break scheduled. Why don't we meet back here promptly at 10:40. We will start up again at 10:40. Thank you.

(Break)

CHAIRPERSON WHITE: Thank you for coming back right on time. As Dr. Cray mentioned, this is an analogy of trying to herd calves to try and get everybody back in here, but I think it shows that we are just a very social group and we enjoy talking about this issue so much. So, hopefully this continues over in the breakout groups this afternoon.

Our next speaker is Dr. Paula --

DR. CRAY: Pointer, pointer.

CHAIRPERSON WHITE: Oh yeah. I am sorry. If anyone has a pointer they could donate for this talk it would be greatly appreciated. The ones we have all of the batteries are dying. Anybody? Marc, do you have one? Thank you.

Our next speaker is Dr. Paula Fedorka-Cray. She received her B.S. in Microbiology from the best university around, Penn State.

DR. CRAY: Yea!

CHAIRPERSON WHITE: Sorry.

DR. CRAY: I like this guy.

CHAIRPERSON WHITE: Masters from North Dakota State University where I also was. Is that a Masters of Administration?

DR. CRAY: Yes.

CHAIRPERSON WHITE: From Johns Hopkins University. And a Ph.D. in Veterinary Microbiology from the University of Nebraska Medical School.

She has been employed by USDA-ARS since 1991 and is currently the research leader of the antimicrobial research unit at Richard Russell Research Center in Athens, Georgia. She is also the coordinator for the veterinary arm of the National Antimicrobial Resistance Monitoring System. Dr. Cray.

DR. CRAY: Thank you. Thank you, Dave. I always have a hard time at football season: half of me is blue and half of me is red for Nebraska and Penn State. North Dakota just gets cold.

I will go ahead and talk here. The title of the talk in the paper I think talks about modeling. But what I am going to do is I am going to show you some of the experiences that we have had.

I have been doing this for 20 years now, looking at vibrio, actinobus solois, salmonella, and campylobacter now in various animal systems. Actually looking at the pathogenesis of the disease and colonization carrier state.

And also here from the laboratory is Scott Ladely. Scott and I have worked together for 15 of those last 20 years and much to his credit he has still lasted, I must be doing something right. He has as much experience doing this as I do and hopefully we would be split up between two different groups.

He is a wealth of information and I would ask him questions also in the hall if you have any after this presentation.

(Slide)

And, before we start I would just like to acknowledge Scott and my collaborators and most importantly the people in the lab who really make all of this happen, who are still back, literally, working as we speak.

(Slide)

Well, I think one of the most important points from this whole talk, if you don't take anything else and you can nod off now after coffee and donuts, is that you have to ask the right question.

And the problem is what is the right question? The right question is the question that you want to have answered. But, the interesting thing about microbiology and animal models is that you can manipulate the system to really achieve most of what you want to achieve.

Therein lies the confounding basis for some of these studies is that now that we have done enough of these, we can challenge and we can set up a system to observe either disease or shedding or colonization or the carrier state under enough different parameters.

It gives us a really good idea then of what might be happening, not only in a challenge, a laboratory design system. But also what might be happening in the field too. And we have carried a number of these studies out into the field so that we have a very good idea of whether there is a synchronization going on between the information.

But again, I think that you really have to sit down and you have to ask yourself what is the right question. And you really want to only ask one question. Because once you start adding more variables in, it becomes very difficult I think to achieve the answer that you are really looking for.

(Slide)

So, where do we look? Some people would say we just want to look in the animal. I think what we really need to do is look everywhere, somewhere, and sometimes nowhere or what we perceive is nowhere. And I think it interesting from the last talk, from Marc's last talk, is that one of the things that I find to be most predictable about microbiology is its unpredictability.

In that, I think that a lot of what you are seeing is that there are these microorganisms that are survivalists and they are creating their own niche in the environment. And so where you might not think to look or where you might think that there is nothing residing anymore, in fact the bacteria are still there.

Either they are viable, but not culturable or they are in such low numbers that we don't have the sensitivity and specificity to find them yet. And we will see how some of that fits in as we go on.

(Slide)

So, one of the first things that we need to go ahead and do is we need to select a target organism. In addition to asking the right question what do you want to test? What kind of target pathogen do we want to look at? Do we want to look at the organism that drug might be indicated for use against?

Are we going to look at a food-borne pathogen? Are we going to look at salmonella or campylobacter or 015787? Are we going to look at a commensal, E. coli? Are we going to look at enterococci? Are we going to look at lactobacillus? Are we going to look at anaerobes? Are we going to look at any of the other aerobes?

And you can develop list, upon list, upon list of organisms that you are going to look for. And all of this is going to be limited by time, money, and expertise of the lab that you actually have built up to look at these model systems.

And, it may come down to a time how what we do in a lot of these situations is that we actually select now, in our studies, many different organisms and we freeze them. You talk about collections, if anyone ever got into our freezers downstairs we probably have 60,000 cultures now in some various states of form.

We also have a lot of frozen feces too. It is not some place I would want to be if we had a power outage and you were stuck in a tornado. But, there are things in the freezer. It is just what is in the freezer.

And what we are doing now is we are trying to develop these studies so that we are actually saving these organisms, taking them from the same animal so that if we want to ask a different question for another organism we can do that from the same test conditions retrospectively. And, if you have the ability to do that, that is one thing that I would encourage you to set up.

(Slide)

All right. We have gone down through here looking at aerobes. Do we select anaerobes, enteric bugs, respiratory bugs, and now do we need to consider mixed infections? I mean yesterday we heard a lot about "take it to the field". Well, the problem with taking it to the field is that you have a whole other complicating set of factors that are superimposed now upon your original design.

And those in particular are mixed infections. Especially with the viral pathogens which tend to relegate then some of the food-borne pathogens to secondary infections. And, it also exacerbates clinical disease and actually outcome. So survivability will be affected in these situations too.

And you can have salmonella, especially PRRS, and we have a paper coming out that clearly demonstrates in Vet Micro in the next month or so, that if you have a PRRS infection with a salmonella infection you are going to increase your mortality about five-fold.

And that would have tremendous impact on what you may be looking for, what you may want to select, and how many animals you may have to select to get the desired number of organisms that you want.

And E. coli also convicts with a variety of viral pathogens and other bacteria too to then be relegated to a secondary pathogen. Now, the other thing that we have observed over time is the effect of the antibiotics on the actual bacteria as we screen for these.

If we look at -- we looked at 420 isolates of salmonella from a study and we looked at the effect of resistance to tetracyclines: oxytetracycline, chlortetracycline, and tetracycline and we essentially saw no difference between the three.

However, there had been reports where if you look at pasteurella or some of the other E. coli or other bacteria and the tetracyclines that in fact you will see a difference in resistance between the oxytets, the chrlortets, and the tetracyclines.

So, in fact if you are reporting out that you just have resistance to tetracyclines, and you are not very specific in your drug, you may in fact be misrepresenting what you are actually going to need to be looking at.

(Slide)

So, if we take salmonella for an example here and we look at three different parameters. If we want to look at disease, then in fact we need to have fairly high exposure doses in a laboratory situation. Typically 10^9 and up. And I liken this sometimes to just giving them a paste. And, it is also a very strain dependent.

And I have UK's story here. And this is not a United Kingdom story, this is a Roy Curtis universal killer story. He has a strain that he calls UK, for universal killer, because he swore this strain would kill anything. And so we said okay, well send it to us because he had never tested it in pigs. This was in my other life in Iowa.

And so we put this Typhimurium into pigs and we thought well, we better do a 10^8 dose so we can just see how devastating it is and then we will knock it down to about a 10^3 dose so we at least have some animals that survive. And we went in the next day and we went down there with all of our necropsy gear on thinking that this was going to be one of those mornings and in fact it looked like we had given them bubble gum or candy.

I mean they were running around. I called Roy and I said you are not going to believe this. We had to send him the isolate back again, but the universal killer apparently doesn't kill pigs. Or these were super pigs. These were Iowa pigs. They are good chops.

But, I think that what it points out is that even within the same serotype that you can have differences that occur and especially between animal species, if we take a Typhimurium from chickens say and we try to infect swine we have a very different outcome then if we take that same Typhimurium and pass it through a pig first.

So, there are all of these confounding factors now when you look at exactly what you are going to try to reproduce the disease with. All right.

Well, then if we just want to set up a model where we look at just shedding and we don't have disease or we have very mild, transient, clinical disease: some febrile response, inappetence, some other things going on. Then we can cut the dose down to a range of about $10^6/10^8$ CFU per pig.

And if we just want to look at colonization for a short period of time, and by short I mean about six to eight weeks and not through to slaughter age, then it was absolutely no disease. I mean it looks like you have done nothing but annoy these guys after you give them the challenge doses.

You give them about a 10^3 and you have very minimal and short-term shedding.

(Slide)

Now, if we look at salmonella heidelberg under these situations and we look at two weeks and six weeks post-challenge, at 10^3, 10^6, and 10^9 dose, and if we look at the tonsil, the ileo colic lymph node, the ileo colic junction, and the cecal contents -- and I just have to say it is so nice to be talking about something besides antibiotics right now. This reminds me of my other life -- that at two weeks from the tonsil and at six weeks we really don't have recovery at a 10^3 dose.

However, these are log 10 CFUs, so we have about a one and one-quarter log, a half a log, now if we boost it up to a 10^9 challenge dose you see we dramatically increase the number of bacteria that can be isolated per gram of tissue. And this holds true now, looking at the tonsil, the ileo colic lymph node, the ileo colic junction, and the cecal contents. These are internasal inoculations and we will go into that in the next couple of slides.

But you can see then that depending upon the tissue that you select and depending upon the time that you are looking you can find very different numbers residing in any of these tissues.

(Slide)

All right. If we have a lab-attenuated strain, I think that typically they tend to be not very virulent. I think that this is part of the problem that we find when people try to repeat our challenge studies and they end up using an ATTC strain.

Or we find out that they have had a strain that they have passed for 52 and one-half years now because Salmon gave it to them, you know, and they really like this strain. Well, it just doesn't work that way. And what you have to do is that you have to pass it back through a mouse or the species that you are working with at that particular time to boost its virulence.

Essentially, I think that what you are doing is you are turning everything back on. You are exciting the bug again and it is up for doing battle now in the host system. The field isolates then we find to be often more virulent. Especially those that we actually take from a disease case.

However, going back to salmonella as an example, we have to look at the serotype considerations. There are more than 2,400 different serotypes and not all of them are equally virulent, although all of them have the potential to cause disease. I think that is a very important point that you don't want to forget.

However, we find that the virulence can differ dramatically within animals. If you had Typhimurium in just about anything at the particular dose you can induce disease. If you have Poona, Poona is very often recovered from exotics. Iguanas love this stuff, which is why I will never have my sons -- well, besides they are ugly. They will never have an iguana in the house.

But Poon is often recovered from children. It is very virulent in children, but it is almost like a commensal in exotics. Okay.

(Slide)

And then you have differences in resistance. And what do I mean by that? Well, if we look at the top 12 serotypes -- I must have been delirious and couldn't count --

you can see that from 1998 we had 557 Typhimurium and on down. So these are the first six and the next six. And those are just the actual numbers that we have out of about 3,318.

(Slide)

If we look at beef on farm or cattle on farm, cattle diagnostic samples, cattle slaughter, chicken diagnostic, and chicken slaughter and those are the total number of isolates we had in each of those groups. We can see then a specificity for some of the serotypes begins to emerge.

Certainly, the chickens at least compared to beef, you will find Heidelberg more often than you will find Derby and you will find Derby more often in swine -- this is swine diagnostic, swine slaughter, turkey diagnostic, turkey slaughter -- then you will Heidelberg.

And then you find some of these other ones mixed in there depending upon the situation. So you do find a significant association with some of these serotypes with some of the species that you are going to be isolating them from.

(Slide)

However, as serotypes vary within each of these production schemes, and I will show that in the next two slides, so does the resistance between these serotypes. Typhimurium regardless of species tends to have the highest resistance associated with it and others would include

Heidelberg and Mbandaka.

While salmonella sero montevideo may show very, very little if any resistance. The same with Poona and all of the ones that you get from the exotics.

If you look at salmonella anatum and agona, which are most often resistant to tetracycline and very little resistance to the other antimicrobiotics. Well, those are the ones that are most often recovered from swine operations, too.

(Slide)

Now, serotypes may also be related to clinical outcome. And we have talked a little bit about that. If we look at a study that we just conducted with poultry and we look at DT104 a sensitive strain versus DT104 a resistant, the penna resistant, the typical. So we have a pan sensitive DT104, then we have a penna resistant DT104.

And we expose these poultry on day of hatch to two seeders that were challenged with 10^8 CFU of either one of these bacteria. We find that although there was absolutely no clinical disease between these groups, in fact the resistant bug is shed in higher numbers, significantly higher numbers, and it also significantly colonizes more birds.

So we have two same serotypes, two same fasche types, but we have a resistance versus a sensitive phenotype essentially and we have a very different outcome when it comes to shedding and colonization.

(Slide)

Now, some of the other considerations that we have to have our genetics. Now we are trying to build the best -- I can't say bug anymore -- but we are trying to build the best animal, all right. We want to have an animal that is resistant to disease, that is resistant to drought, resistant to McDonald's burgers, resistant to a lot of different things.

So, you have animals now that you are actually trying to incorporate some of these resistant strains into. However, the problem with salmonella is that it is ubiquitous and it is very, very hard to find any animals at all that aren't colonized with salmonella. Especially when you look at conventional systems.

So, if we try to -- one of the things that was pointed out in several of these presentations was that you want to have animals that are free of salmonella. That is no easy task. It took us several years to develop a model where we have actually been able to routinely now take piglets and maintain them free of salmonella.

But, in these early studies we looked at a group of 43 sows and we had two sows positive for salmonella before farrowing. Seventy-two hours after farrowing you had 27 of the 43 sows that were farrowing.

And then you always have your outlier. You had one that was negative, negative. Obviously, she liked being pregnant and having kids. But here you have 38 sows and we had six that were positive after farrowing and we only had one that was negative, negative again.

If we wean these pigs at 10 to 14 days of age, especially from sows that we know are negative from salmonella, then we can typically maintain them free of salmonella. But, what happens is if you just wean at 10 to 14 days of age and take it from this type of scenario, we ended up with 41 animals out of 407, or about 10 percent, that were positive.

Now, not all of these serotypes interestingly matched the sow. So the question is where were they coming from? Well, they were probably coming from the environment or they were in such low numbers that we weren't able to recover them in the first place.

Now, we also have to look at the age of the animal when we look at the animal models because we know that for salmonella especially less than four to six weeks of age it is very difficult to reproduce disease in a lot of situations. And that is because you have the maternal antibody coming through.

However, the most susceptible age is six to eight weeks of age. And then you come into another window then of about 8 to 10 weeks of age where literally you would have to give them a paste in a lot of instances to get them sick again.

So, it is very, very important to look at the animals that you are using in any of these experiments. How you are selecting them and are they truly free of what you want to look at. And one of the other organisms I think that is going to be just horrendous to try to work out a system with is campylobacter.

(Slide)

That is why I am using salmonella in these slides. So, if we look on the farm then at just some other confounders, well what are we going to find in a typical farm situation? Well, we went on to this one farm, and actually we repeated this twice, and if we looked at the farm and we actually did field environmental samples.

So we took 150 samples every time we went out. We also looked at tissue. We necropsied 10 pigs, all the way through slaughter. And if you don't think necropsying 250 pound pigs in the middle of July in Iowa is a picnic, boy, come see me this summer!

And what we saw was is that we had these seven serotypes that we were able to recover, but not all the time. We never saw S. agona until nine weeks of age and that persisted through until slaughter. We saw Agona sporadically, more consistently missing the ninth week. Berta we only saw once. Brandenberg we saw through nine weeks. Johannesburg we did not see until slaughter. Monte Video, 18 weeks and Worthington one, nine and 18 weeks.

Now interestingly, we also followed these carcasses into the slaughter plant. And if we did carcass swabs and if we looked at their lymph nodes that we were able to take, all of the carcasses were negative.

So, I think that this tells you that when you go into a field situation that you are going to have a lot of different scenarios and when you look at trying to track your own organism or a marked organism through the system, I think that you are going to encounter a monumental task when you go ahead.

It is not impossible, but you have to be aware of a lot of the other confounders because if you had a plate that was mixed at any one time with two to four different organisms, you are going to have to pick many more colonies to try to find what you are going to need to be looking for.

(Slide)

Now, one of the favorite areas that I have is the rod of inoculation. Some of the work that we did looking at internasal versus the gastric, and this gastric was actually a gelatin capsule that -- a big, big gelatin capsule -- that we filled with feed and then we inoculated it with salmonella, put the top back on and you can let it dry down and it hardens.

You can still recover the salmonella because it desiccates quite well. And if you shove this down, and so you are inoculating it much like they would be taking up feed, versus an internasal. And historically salmonella is thought of as a fecal/oral contaminant.

Well, we could find with our internasal rod of inoculation that we have a more significant clinical outcome. That humoral immunity is affected. Although we have an IgM and an IgG response the gastric tends to produce a greater IgG. We don't see IgA with either one of those.

Cellular immunity -- the B cell response is greater with internasal versus the gastric. However, shedding levels will be significantly higher especially in the first three weeks of the experiment. Whereas the gastric, we are only going to see shedding typically for the first three days at a high level, and then it will decrease over time and you will just find sporadic shedding.

Tissue distribution tends to be much higher in an internasal inoculation. In gastric it tends to be much lower. The actual numbers in the tissues tend to be two logs higher from an internasal inoculation versus a gastric.

(Slide)

Now, why do I bring this up? It is partly because I don't necessarily think that we can look at salmonella in the typical boxes of fecal/oral pathogen. Especially with our confined facilities. Especially with the evidence that is been presented by ourselves and other people, including Cliff Wray from the U.K. and deJong from the Scandinavian countries that aerosol transmission is highly probable.

If we look again at an internasal inoculation, this time with Typhimurium, and a trans-thoracic where we actually went in between the third and fourth rib, directly into the lungs, and then we look at our typical gut challenge right into the stomach again with our pill popper, we can see that in three hours the tonsil has 4.6 logs and 3.3 logs in the trans-thoracic challenges. Nothing now in the gut.

The gut issue, the ileo colic lymph node is negative at three hours, but at six hours it is positive. That is a rapid dissemination throughout the host. And what we did was we actually esophagautimized these pigs so we took out any possibility that they could have received any type of gut challenge with our internasal model here.

And this suggests to us that in a transport situation where you are going to commingle pigs at any point in time and they have a possibility of being in contact with any other serotype, there is a high likelihood that they will in fact become colonized, at least to low levels then with another serotype.

If we look at 18 hours now, we can see that our gut is now just becoming positive with non-quantifiable levels. But, we are still, especially with the trans-thoracic at 4 and 5 logs of salmonella that we can recover. I think this is something else that you have to consider though when you are setting up your challenge models.

(Slide)

Now, you can also look at fasts: no food versus reduced food. This typically occurs only after clinical illness has been initiated. I mean pigs, chickens, everybody they all eat -- I will leave that comment out -- and what we see though is that if you want to mimic some things then I can't quite see why you would want to fast because you really don't see that.

We don't stop eating, even as humans, until we are slightly depressed, feeling a little yucky, taking some Tylenol. And that is the same scenario that is happening in an animal situation, too.

So, if we don't fast pigs, then do we have a more natural exposure? I would submit to you that we probably do. Some people will do a stomach neutralization too and I would contend that that is even -- I mean you don't go in and take, before you are going out to eat, and say okay let's take a dose of sodium bicarb. We are going to go out to eat and see what we come home with.

That is not a natural scenario, in most cases I would guess. And so I would say that no neutralization is probably the way to go too. So, if you read a lot of the historical references and you are reading a lot of the outcome based on animal models that have been presented, you have to look and you have to evaluate exactly what they were doing and were they manipulating the situation in any particular way to influence outcome?

(Slide)

All right. So not only do we have the internasal and the trans-thoracic and the gut challenge and your typical per OS challenge, what we like to use now are seeder animal models. Which is, what I consider to be one of the most natural ways.

And again, we are still taking a guess. But, what essentially we like to do is we like to take our pen, our unit, that we are going to challenge. We like to challenge one or two other animals or some other ratio that we pick. And we typically challenge those with our organism and then we introduce those birds or pigs or cattle or whatever into our herd or unit situation.

And we look at the dissemination of the bug then amongst the population. And we believe that that is going to simulate in a much more natural manner then exactly what would be happening under field-type conditions.

And Jeff Gray published a very nice paper a couple of years ago which clearly demonstrates that by using this type of model system that we can mimic a lot of the experimental and in fact the field conditions that are going on.

We find that the serologic response is often the same, especially with our challenge systems that you would be doing a direct challenge with every animal. And the bacteriologic response is parallel.

And we can evaluate this spread within both the group and the environment then. Because, not in all situations are we going to find that all of the animals will become infected. We always have those outliers in any one scenario.

(Slide)

So, the question then becomes if we set up a situation and we have done some looking at the effect of drugs, is when do we treat that? Do we treat when we see clinical illness? Well, what if we can't induce clinical illness? Do we treat when we would expect clinical illness to occur, which is typically 24 to 48 hours after exposure to the salmonella?

These are questions that you have to ask. Now, how many times do you treat? Do you treat by label indications? Or do you mega treat? I would submit to you that if you are going to stand out there and inject or expose drugs all of the times that you are going to influence the outcome of the resistance population.

So, should we be looking at setting up models where we are only looking at it under label indications. And then perhaps doing more screening of the environment which may in fact be the seeder population for other naive pigs that you may be bringing in. And that brings us to the re-exposure route.

The environment plays a critical role, not so much in our isolation units, but typically on the farm where we have a fecal and/or litter build-up. It also raises the question about our naked DNA running around there or laying around there or whatever around there, being available for incorporation into all of the other bacteria that might be around. So these again are all different parameters that we have to look at.

(Slide)

Well, since I forgot to bring the exact numbers with me and I couldn't remember, I did remember the exact simulation, though. And what we did was we looked at S. Heidelberg and we treated, we exposed two groups: the red and black and the yellow group to S. Heidelberg on day zero to about a 10^9 dose. And we did start to see some clinical illness at about 48 hours

So we treated which is what the stars indicate on days two, three, and four with either drug A or drug B. And what we found was that with drug B, by day five we absolutely had no shedding levels whatsoever. We couldn't find it. And we looked extensively for any bacteria that we could find.

And now our culture methods I can tell you are sensitive down to 1 CFU per gram. And we couldn't find any salmonella on day five. But by day six, or 48 hours after we stopped treatment, then we started to see these levels come up. So this very clearly demonstrated the differences in reducing a pathogen load, but not necessarily eliminating the pathogen from the environment. And these are the types of scenarios that you can probably expect with some bug drug combinations.

(Slide)

Now, having sat through a whole day's worth of talks, one of the nice things about Powerpoint is that you can begin to critique everybody else's and no one gets a chance to critique yours. So, yesterday a comment was made that, you know, do you look at a single drug? And well, I would submit to you that resistance to a single drug is just that, it is resistant to a single drug.

And, we now have the dynamics of multiple resistance in our midst that we have to consider, because we really don't know what that trigger is for initiating the development of a cassette or the incorporation of a cassette or how many drug-resisted genes will be incorporated in any one cassette.

So, even though we look and we say, well this is going to be relatively innocuous because we may only have low levels of resistance that we are evaluating, we can't be remiss and at least think at the back of our mind that in fact any particular drug or disinfectant or metal, something is going to act as a trigger for setting off this movement or incorporation of other genes into the chromosome or between different bacteria.

And again, I think that goes back to the survival mechanism with bacteria. They simply exist to survive.

(Slide)

So, what are we measuring? We are measuring clinical disease. Is that what we want to measure as an outcome? Do you want to measure performance? Which is a totally different question. Do you want to measure average daily gain? Do you want to measure days to market? Do we want to measure shedding?

Do we want to measure this elusive pathogen load that can change depending upon what time of the day? We know that typically animals have cycles too. They don't take U.S. News and World Report into any corner or anything, but they have different times of the day when we find that they shed higher numbers too.

How many people are you going to put out in the pen sampling over a period of time? And all of these different parameters, asking the question what are we going to measure will mean that we have to have different approaches to setting up the models.

(Slide)

What do we test? If you do direct catch, you better have significant back insurance for the person that you are out there asking to do these direct catches. It is much easier to do a direct catch from cattle and swine than it is from chickens.

So, you really have to think about what you are asking for over time. This is where graduate students come in and be very invaluable. I hope there are none out there.

All right. If we look at environmental samples then we have to ask ourselves are we picking up extraneous contaminants and how do we sort those out? Or, were they really just there anyhow and are they going to have a